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Review

Molecular biology approaches to control of intractable weeds: New strategies and complements to existing biological practices

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ABSTRACT

Molecular genetic tools and concepts are in relentless and continuous development, affecting every field of biology. Biological control of weeds, an applied science with over a century of history, is no exception. There are many examples of successful biocontrol of weeds, in some cases quite spectacular. However, biocontrol is not applicable to every weed challenge, in large part due to the limitations of available biocontrol agents. Indeed, one cannot realistically expect that naturally occurring agents have serendipitously evolved to meet all of man's weed control needs. In cases where agents are lacking, molecular biology may be able to improve biocontrol, reduce dependence on chemical herbicides, and control intractable weed species. In theory, any biological weed control strategy could be improved through molecular biology, whether by improvement to a biocontrol agent (e.g. increased lethality, improved specificity, ability to work in concert with other pest management tactics), improvement of the crop (e.g. improved weed-inhibiting properties), by increasing knowledge about the evolution of the target and its natural enemies to assist the search for and selection of classical biocontrol agents, or even by genetic manipulation of the target weed itself. Molecular genetic technology can enable novel strategies that would not be possible in its absence, as well as allow weed researchers to adapt molecular solutions from other fields, including biocontrol of other pests or fields only remotely connected to weed biocontrol. This paper will review concepts of weed biocontrol and molecular biology applications to weed control, as well as propose novel weed-control strategies.

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1. Introduction

The continual invasion of non-native weeds into new environments is inevitable considering the continuous expansion of human movement, migration, and global trade. Indeed, in most countries the intentional introduction of plant species for forage, agriculture, and horticulture is less strictly regulated than, for example, the introduction of classical biocontrol agents, despite a well-documented history of many such plants later becoming invasive weeds [1]. The demand for measures to efficiently control existing and anticipated invasive weed challenges, combined with increasing worldwide sensitivity to environmental propriety, ensures that biocontrol in some form will play an important role in weed control in the foreseeable future.

Different weed challenges call for different control strategies, whether biological, chemical, cultural, or a combination of these [2,3]. A biocontrol strategy may be warranted because of concerns about environmental effects of herbicides or mechanical control, lack of efficacy of other methods, or simple economics. Biocontrol of weeds can be separated into three distinct strategies: conservation, inundative, and classical. These strategies apply to different weed challenges depending on the setting (e.g. range, row crops, urban), the extent of the infestation, and the biology of the system. Conservation biocontrol refers to situations in which a biocontrol agent is already present in the range of a weed and is able to control the weed but requires assistance in the form of cultural practices or pest management decisions that enable the agent to thrive [1,4]. Conservation strategies are less common in biocontrol of weeds than in biocontrol of insects [1] but examples do exist [4].

Inundative biocontrol involves the release of large numbers of an agent at a time when weed populations are expected to escape control or exceed a critical economic or competitive threshold. When the agent is already present at a level that does not provide adequate, continuous control of the target, this strategy is known as augmentation biocontrol [4]. In general, inundative biocontrol relies on the released organisms themselves to control the target without any expectation of continued control by future generations of the agent [5]. In an inundative strategy the agent may need to be released or applied several times during a single crop cycle in the event of re-growth or re-emergence of the target weed. Thus, inundative strategies typically apply to relatively high-input systems.

Classical biocontrol (sometimes referred to as inoculative biocontrol) refers to the practice of identifying co-evolved natural enemies from the native range of a target weed species and releasing them into the invaded range to reduce the presence of the weed to acceptable levels [1,4]. In classical biocontrol, the agents are expected to reproduce and proliferate on the target weed and disseminate throughout its invaded range, reaching an ecological equilibrium with the target weed and providing continuous, perpetual control. Successful control depends almost wholly on damage caused by the descendants of the released individuals rather than by the released individuals themselves [5]. Classical biocontrol is generally practiced in low-input systems.

Biologically based weed control can also take the form of weed-resistant properties in crop plants, akin to host–plant resistance vs. insect and pathogen pests. Allelopathy, the production by a plant of secondary metabolites that inhibit growth of nearby plants, is a phenomenon that has been studied for its potential utility in weed control for many years [6]. Despite its long history, little progress has been made in incorporating allelopathy into mainstream weed management programs due to a failure to provide adequate weed control while maintaining other agronomic qualities of the crop [7]. Recent reviews provide a comprehensive treatment of the subject [7,8]. Research on utilizing allelopathy as a mechanism of "resistance to weeds" in agricultural systems focuses principally on exudation of phytotoxic chemicals from the roots of crops [9], although it would be possible in perennial crops to realize allelopathy via fallen leaves. In annual cropping systems, successful transmission of an allelopathic effect at a sufficient distance through the soil to produce an economically significant reduction in weed growth and competition poses an important challenge to the development of this technology. As such the greatest immediate promise for allelopathy may be in protecting crops from parasitic weeds such as witchweed (*Striga* spp.) or broomrape (*Orobanche* spp.). These weeds make direct contact with their hosts and therefore may be particularly susceptible to allelochemicals produced by the host plant. Crop-based biological weed control strategies are inherently amenable to enhancement using molecular technology. Approaches that would not be possible in the absence of such tools, such as genetic manipulation of the target weed to facilitate its own control have also been proposed [10].

This paper will review inundative and classical weed biocontrol concepts and other biologically based weed management strategies and discuss current and proposed molecular approaches to intractable weed control that seek to either improve existing biologically based practices or suggest new ones.

2. Inundative biocontrol

2.1. Bioherbicides

In weed biocontrol, inundative biocontrol agents are typically microorganisms formulated as "bioherbicides" that are proposed as environmentally benign substitutes for chemical herbicides in cropping situations [11]. Early research and field successes of bioherbicides, particularly mycoherbicides, suggested that an

overwhelming number of spores of a native, target-specific fungus could be used to turn a normally endemic pathogen into an epidemic [12]. Consideration was also given to addressing other factors that may prevent epidemics in nature, such as genetic diversity in both the weed and the fungus. In addition, the early focus was on specific weed-pathogen combinations [12]. A more recent treatment of the subject [13] focused on increased virulence as a more important limiting factor to creating artificial epidemics than extreme numbers of spores, and proposed a greater role for broad-spectrum bioherbicides. Whatever the case, a bioherbicide must be properly integrated into the overall pest management program and tailored to adequately control the target weed, whether through the serendipity of natural selection [14–16] or through the use of molecular biology or other tools. For example, the bioherbicide may need to be tolerant of selected pesticides depending on the needs of a given cropping system [17].

Microorganisms have a somewhat mixed record as bioherbicides. Many pathogens have been studied as potential bioherbicides for inundative biocontrol. However, more than a quarter century after registration of the first bioherbicides [15,18], the current state of the art has not resulted in widespread adoption of such products for large-scale weed control [1,11,19]. Nonetheless, one can assume that there will be a viable market for bioherbicides that do their jobs safely, effectively, and economically, regardless of their origins. The paucity of commercially available bioherbicides that currently fit this description indicates that improvements are still necessary across the board in the field of bioherbicides. Molecular biology technology may be able to provide some of the needed improvements.

Virtually any heritable trait of a bioherbicide can be enhanced or suppressed using molecular methods, given a sufficient amount of information about the genetics of the system. The idea to manipulate microorganisms for use as biocontrol agents is not necessarily a new one [12,20,21] and over a decade has passed since the first fungi were genetically transformed for enhanced control of insects [22] and weeds [23]. However, despite advances in the production of genetically enhanced bioherbicides [19,24,25], the fruits of this technology have yet to become an important presence the marketplace.

2.1.1. Useful bioherbicide traits

While a narrow host range is essential for a classical biocontrol agent (discussed below), in an inundative strategy it may not be [1,5]. For example, the fungus *Sclerotinia minor* will attack many dicotyledonous hosts and has been developed as a bioherbicide to control broadleaf weeds infesting turfgrass [26] as a sort of "biological 2,4-D." Other broad-range pathogens, such as *Myrothecium verrucaria* [27] and *Phoma macrostoma* [28] are in development to become bioherbicides. Such broad-spectrum bioherbicides are more useful than highly host-specific agents in situations where a complex of weed species must be controlled, although in cropping systems they are limited to use with those crops that are not susceptible to attack by the bioherbicide. Mixtures of host-specific bioherbicides have also been proposed for controlling complexes of weed species [29].

Plant–pathogen interactions are complex and in many cases poorly understood, which can lead to unpredictable (and therefore unreliable and unmarketable) results when pathogens are utilized as bioherbicides [11]. In addition, co-evolution of pathogens with their hosts seldom produces the type of quick kill or "knockdown" that weed managers seek, for the simple reason that such a knockdown is generally advantageous to neither the pathogen nor its host. In broad evolutionary terms, a host-specific pathogen that is too virulent risks eradicating its own host and thus driving itself extinct [19]. Thus knockdown, or hypervirulence, is a common character targeted for enhancement [19]. This capacity for rapid kill is essential in a bioherbicide, both to minimize the duration and amount of weed competition with the crop plant and, from an agronomic standpoint, to compare favorably to the chemical herbicide that it may seek to replace. Clearly the bioherbicide must also not harm the crop plant and should not persist in the environment after control is achieved in order to avoid adverse effects to susceptible rotation crops or other non-targets. Application via machinery that customers are likely to already use (e.g. chemical pesticide applicators) is another useful trait for a prospective bioherbicide, however if the product is effective and economical, customers will adapt to new modes of application.

The ability of a pathogen to penetrate the defenses of the healthy, living target plant is another desired trait in a bioherbicide and is common in fungal plant pathogens. However, hypervirulence is not commonly seen in these fungi since in nature invasion of healthy plant tissue allows the fungus to take advantage of a nutrient resource that is unavailable to common saprophytic microorganisms that colonize only dead or wounded plants. In killing its host too quickly, a pathogen would squander this nutrient advantage by inviting competition from saprophytes. Bioherbicides that can penetrate their hosts' defenses will thus likely need enhanced virulence to kill their targets quickly enough to be useful as bioherbicides. Those that cannot invade healthy tissue may require application protocols that wound the target plant [30].

2.1.2. Bioherbicide enhancement

2.1.2.1. Hypervirulence. Transgenetically engineering virulence-enhancing factors into candidate bioherbicides is a straightforward approach to increasing hypervirulence and there are several options available that present potential solutions. Gressel and colleagues [19] have tested a number of genes encoding virulence-enhancing factors that they termed either "soft" (i.e. natural plant compounds already present in the human food supply and generally regarded as safe) or "hard" (e.g. potent phytotoxins). Soft genes were identified based on inferences into why certain observed mutations in plant pathogens had resulted in loss of virulence. Thus far, tested soft genes have included those encoding auxins, pectinase, expansins, and oxalate synthesis [19,24]. Hard genes tested include those encoding production of the fungal phytotoxins NEP1 and cerato-platanin [19,25]. Tests have been conducted using several different target-specific pathogens and combinations of soft and hard genes have also been tested. Enhanced virulence has been observed in some but not all instances [19].

Another aspect of engineering hypervirulence to target specific weeds is to engineer commonly studied, model pathogens with genes encoding host-selective phytotoxins or virulence factors [31,32] procured from plant pathogens that are specific to the target weed species. This strategy would be advantageous if the target-specific pathogen that is the source of the toxin or factor is an insufficient bioherbicide by itself or is difficult to culture or transform. In addition, the use of host-selective toxins or virulence factors might be considered "more host-specific" than an engineered host-specific pathogen in the eyes of regulatory authorities, as it would not be possible for a host-selective toxin or virulence factor to change targets. Escape of the gene construct to wild pathogens would have less potential for agricultural harm because of the host-selective nature of the gene product. Virulence-enhancing factors tend to be simply inherited [32] whereas host-selective toxins are usually secondary metabolites [31] and therefore may be challenging to produce transgenetically. In addition, host–plant responses to host-selective toxins are

frequently single-gene interactions [31], which could limit their effectiveness in controlling weed populations with allelic diversity at the response locus, a phenomenon that is well known in crop–pathogen interactions [32,33]. Most known host-selective toxins and virulence factors affect crop plants [31,32], so a discovery phase would be required to identify such elements that are specific to a chosen target weed. Selective toxins may also exhibit activity against a small range of unrelated plant species [34].

Another interesting strategy has been proposed to enhance virulence in bioherbicides by selecting or generating variants of target-specific plant pathogens that overproduce amino acids that are toxic to the host plant [35,36]. This strategy is an elegant biological solution that in its simplest form does not require extensive molecular machinations but does rely on (a) the existence of severe to lethal sensitivity in the target weed to certain amino acids and (b) the generation or existence of selectable mutants of target-specific pathogens that produce the desired amino acid in quantities large enough to inhibit the weed [36]. Frenching disease in tobacco [37] is an example of this phenomenon occurring in a crop plant and sensitivity to particular amino acids has been noted in several important weed species, including *Cannabis sativa, Cirsium arvense*, *Convolvulus arvensis*, *Orobanche ramosa*, and *Poa annua* [36,38]. Overproduction of key amino acids has been reported as a means of enhancing the virulence of weed biocontrol agents [36]. Persistence of amino acid overproducing mutants in the soil could pose risks, with agricultural implications if crops subsequently planted to the same land were sensitive to the amino acid in question. Amino acid overproducing prokaryotic bacteria would present the risk of horizontal transfer of genes conferring this trait to prokaryotic crop pathogens.

2.1.2.2. Understanding fungal toxins. Among the fungi, a great variety of toxic metabolites are known with toxicity against many different groups, including plants and mammals. Some fungi may produce toxins affecting different kingdoms, including individual toxins affecting multiple kingdoms. Two such fungi, *Fusarium tumidum* and *Myrothecium verrucaria*, are promising bioherbicides that produce both phytotoxic and mammalian cytotoxic metabolites, including some with dual activity [39,40]. Use of either of these fungi as a bioherbicide without the possibility of separating phytotoxic and mammalian cytotoxic activity would raise both social and environmental safety concerns [27,41]. However, recent research on aflatoxins, a class of mycotoxins produced by *Aspergillus* spp., that are highly toxic and carcinogenic in humans, has produced the full sequence of an aflatoxin-biosynthesis pathway in *A. parasiticus* [42] as well as exciting genetic information explaining why some congeners (including *A. sojae*, the common fermenting agent of soy sauce) are nonaflatoxigenic [43]. Such information may prove useful to researchers studying promising bioherbicidal fungi that produce mammalian toxins in addition to phytotoxins [44].

2.1.2.3. Crop-bioherbicide combinations. Genes for non-selective phytotoxins could be of benefit to generate broad-spectrum bioherbicides via broad host-range microbes [11]. The availability of genes conferring production of as well as resistance to such toxins leads to the possibility of transforming crop plants with a resistance gene corresponding to a phytotoxin transgenically expressed in a microbe [45], which would then be sprayed over the crop, emulating the transgenic herbicide-resistant crop paradigm used for many row crops [46]. A similar proposal is to identify resistance genes to a broad-host-range plant pathogen, then insert them into crop species, using the pathogen as a broad-spectrum mycoherbicide [47]. In such a system, virulence of the pathogen might also need to be enhanced and persistence in the field curtailed.

2.1.3. Preventing gene flow out of transformed organisms

As with any project that proposes the release of genetically transformed organisms into the environment, it is essential to prevent gene flow from these organisms to wild relatives, particularly if the transferred genes are likely to confer a competitive advantage to wild organisms. To prevent such gene flow, additional traits that will be desirable in enhanced bioherbicides include lack of persistence in the field, lack of reproduction, lack of off-site dispersal, and lack of recombination ability with other microbes. In addition to increasing the risk of unwanted recombination, persistence in the field also raises the possibility of competition in subsequent applications between freshly applied bioherbicide and the persistent descendants of previously released microbes, which are likely to lose virulence in post-release generations [48]. In essence, the ideal bioherbicide would act as a kamikaze [49], target-specific vector of a hypervirulence factor, killing the weed and perishing upon completion of its mission. These traits would be particularly important in broad-spectrum bioherbicides. Mechanisms proposed for prevention of persistence, dispersal, and movement of genes from genetically modified mycoherbicides to wild organisms were covered in depth by Gressel [50]. They include the suppression of spore formation through either transgenic or mutagenic means (e.g. via suppression of melanization), formulation of the mycoherbicide as dehydrated mycelial fragments rather than as spores [51] (where feasible), use of existing inducible-sterility technology [52], or incorporation of genes flanking the bioherbicidal gene that would be neutral or favorable to mycoherbicidal activity but deleterious in other species.

2.2. Arthropod agents

Augmentation strategies with arthropod agents are sometimes practiced in situations where the target weed is intermittently dispersed over large distances [53], exist in mixed habitat unfavorable to agent dissemination [54,55], or where the agent simply disperses too slowly without redistribution efforts [56,57]. Such arthropod augmentation strategies can also be thought of as classical (inoculative) strategies on a limited scale, since control is generally predicated on establishment and feeding by the descendants of the original release populations rather than by the released individuals alone. Arthropods are more commonly used for inundative control of other arthropods than for inundative weed control. However, examples exist of arthropods being used as truly inundative weed biocontrol agents (i.e. large populations reared and released specifically for control of weed populations due principally to the first feeding generation of the released organism), including for control of nutsedge (*Cyperus* spp.) [58] as well as the parasitic weeds dodder (*Cuscuta* spp.) [59] and broomrape [60]. Such releases typically require large, permanent investments in insect rearing facilities, which may preclude their widespread use.

2.2.1. Enhancement of arthropod agents

Attempts have been made to improve the efficiency and lethality of an insect inundative weed biocontrol agent by coating larvae with herbicide prior to release [61]. Molecular tools might also be of use in attempts to increase the efficacy and lethality of arthropod agents, as well as other traits, such as reducing dispersal so that the agents remain close to where they are applied, prevention of reproduction (such a trait would need to be inducibly deactivated in order to allow mass rearing in the lab), resistance to

selected insecticides, or increased voracity (e.g. via induction of supernumerary larval instars [62]).

2.3. Other proposals

Other applications of molecular biology to inundative weed biocontrol have been proposed. For example, manipulation of ruminant gut microflora to better digest defensive compounds of certain weeds has been proposed to allow grazing on land invaded by toxic weeds by otherwise unsuitable species of livestock [63] (i.e. mammalian inundative weed biocontrol agents). Use of virus vectors to express target-specific genes has been proposed to induce gene-silencing in crucial components of the weed cellular machinery [64,65].

3. Classical weed biocontrol

Key characters of successful classical weed biocontrol agents include narrow host range (ideally limited to the target species), persistence in the environment, and the ability to reduce target populations to sub-economic levels over the course of generations. Once released, an agent is expected to establish permanently in its new environment, so host specificity is particularly important in classical biocontrol, especially with regard to non-target plants of economic or ecological importance. The risk of non-target attack is weighed against the expected benefit of controlling the target weed, as compared to an alternative practice or maintaining the status quo [1,66]. Such risk–benefit assessments are a primary tool of regulatory authorities in the field of classical biocontrol and (unlike the host-ranges of the biocontrol agents they assess [67,68]) they evolve continually with the changing demands of the societies they serve.

The first intentional releases of classical weed biocontrol agents occurred in the first half of the 19th century in India [69]. Goeden and Andrés [4] provide a comprehensive catalogue of classical weed biocontrol successes that followed over the next century and a half. To date more than 200 plant species have been targeted for classical biocontrol worldwide involving both arthropod and microbial agents [70].

3.1. Advantages and disadvantages of classical biocontrol

Key advantages and disadvantages of classical biocontrol include the following [4]. Advantages: (1) Under favorable circumstances agents can reproduce and disseminate throughout the range of the target weed; (2) proper host-specificity testing of agents establishes non-target risk; (3) classical biocontrol can succeed in situations and on scales that would be wholly unfeasible for chemical or cultural control [71–74]; and (4) the system and its agents are energy efficient and biodegradable. Disadvantages: (1) Once released and established in a new environment, an agent cannot be "unreleased" or called back (although biocontrol may be attempted, in turn, on a rogue agent); (2) host specificity may be disadvantageous in systems with multiple weed species that must be controlled; (3) discovery, development, and establishment of an agent and eventual control takes time (on the order of 10–20 years); (4) non-target effects, although rare and usually minor compared to the benefit of controlling the target [1,75], are possible and unpredictable (particularly indirect effects [76]); (5) establishment and impact of an agent in the introduced range is unpredictable and in no way guaranteed.

There is no more elegant or economical weed management option than an effective classical biocontrol agent [77,78]. A recent comprehensive economic assessment of 32 Australian weed classical biocontrol programs from the past 100 years [78] showed an overall return on investment, including failed programs, of >23:1. This indicates quite clearly that classical biocontrol is a worthwhile weed control option for appropriate targets.

Unfortunately, classical biocontrol is not an appropriate strategy for all weeds – for example, of the 76 "World's Worst Weeds" of Holm et al. [79], only 24 have had classical biocontrol agents released against them [70] – and it is not effective for every targeted weed [70]. Lack of sufficiently host-specific agents is an important shortcoming, particularly for target weeds in taxonomic groups with many closely related, economically important plants (e.g. Poaceae). However, among the disadvantages listed above for classical biocontrol, perhaps the most important is the last, particularly if the result is complete lack of establishment or impact and failure to reduce target populations (i.e. zero return on the research investment). Lack of establishment or impact by the agent in the introduced range can occur as a result of any number of biotic or abiotic factors in its new environment that may differ compared to its native range (e.g. climatic factors [80,81], photoperiod [82], predation or parasitism [83,84], host–plant resistance in the weed population [85]).

3.2. Rates of success and failure of classical biocontrol programs

Approximately 60% of biocontrol agents released worldwide successfully establish [1,69] but the more important figure is how many targeted weed species are ultimately brought under control by their agents, regardless of how many releases are made. Data regarding the degree of success of weed biocontrol programs can be subjective and difficult to quantify [1]. However, data complied from classical weed biocontrol programs in Australia, Hawai'i, and South Africa [1,78] (n = 76) show that control was not achieved (i.e. net loss or zero return on investment) for approximately one-third of targeted weeds. The failed projects in Australia averaged 10.7 years of group research invested [78], so failure cannot be ascribed to lack of effort.

3.3. Molecular assistance to classical biocontrol

Classical biocontrol programs are very large undertakings requiring substantial investments in time, labor, and money, with the knowledge that at least five years is likely to pass before the first agent is ready for release and 5–15 additional years may be required to see widespread results (although quicker results can occur, particularly in aquatic or tropical systems) [M. Julien, personal communication]. In addition there is a real risk that no agents will ultimately be effective. In recent years, particularly in countries with established histories of classical biocontrol implementation and success, classical biocontrol has become an increasingly prevalent option to consider alongside other practices in integrated weed management programs. However, due to its cost and long incubation period, classical biocontrol has often been practiced as a "last line of defense," after it has become clear that the target weed has escaped economical, acceptable, or logistically feasible control by common chemical or cultural methods, and is spreading unchecked. Molecular approaches may be able to complement or provide alternatives to classical biocontrol in cases where it is not effective, although like classical biocontrol, some such approaches may require high initial investments without guarantee of success and thus might not be considered until other measures fail. However, molecular approaches are already increasing knowledge about target weeds and their biocontrol agents (and candidate agents), thereby enhancing the applicability of classical weed biocontrol.

3.3.1. Genetic fingerprinting

Genetic fingerprinting and phylogeographic studies can help direct searches for biocontrol agents by matching genotypes of invasive weed populations to their places of origin in their native range and by explaining variability in attack by given agents on genetically distinct populations of the target weed. Records of such variable attack include insect [85], microbial [86], mite [87], and mammalian [88] agents. Other genetic fingerprinting applications to classical biocontrol include species identification from agent specimens or samples that are not typically suitable for taxonomic identification (e.g. insect larvae [89], pupae, eggs, or even frass [90]; asexual stages or strains of fungi [91]); speculation about the center of origin of the target species [92]; tracing the origins, introduction, and spread of weed invasions [93]; taxonomic separation of host-specific agents from species complexes with different host ranges [89,94]; identification of novel hybrid populations of the target species in the invaded range [95]; tracking released agents for safety, efficacy assessment [96], or proprietary [97] reasons; and ensuring quality control of biocontrol agents prior to release [98]. A recently launched, interactive, online, taxonomic, genetic fingerprinting project [99] also has applications to classical biocontrol, particularly regarding taxonomy of candidate agents. Similar tools are likely to be developed and proliferate.

3.3.2. Genetic enhancement

As with inundative biocontrol agents, research on genetic enhancement of classical biocontrol agents to address whatever shortcomings they might have (e.g. lack of establishment or impact) should be within the scope of current technology. One can envisage agents enhanced to be more persistent in a new environment (e.g. cold- or heat-tolerant; altered diapause, sporulation or germination cues; suppressed diapause or sporulation), to have narrowed host ranges, increased dispersal ability, or to be more virulent or voracious (e.g. insects with decreased nutritional uptake from food, requiring more feeding per individual). Genetically modified agents are being actively studied for biocontrol of invasive vertebrate pests in Australia [100] and the use of transgenic technology in weed biocontrol has been favorably considered there [101]. However, no classical weed agents have been specifically proposed for genetic enhancement. Certainly, due to the perpetual nature and active dispersal of classical biocontrol agents, neighboring countries to any nation proposing such a project would want to be involved in the decision-making process [102]. For the same reasons, techniques for preventing gene flow out of an enhanced organism would need to be well proven (as discussed above). In addition, arthropods, particularly holometabolous insects, can have very complex life cycles and alterations to traits such as diapause may have important side effects. Nonetheless, among other potential applications, if it were possible to reliably limit the host range of an agent through genetic modification and to ensure that inserted genes would neither fail nor spread to other species, one could dramatically increase the number of weed species that are susceptible to classical biocontrol, especially in the family Poaceae. A comparison of sympatric, conspecific populations of grass-feeding herbivores with divergent host preferences [103] or other similar systems could lead to the discovery of such host-specificity genes.

4. Manipulation of crop species for weed control

4.1. Allelopathy

Molecular genetic tools enable rapid selection for and manipulation of pest-resistance traits in crop plants. Strategies for crop resistance to insects [104–106] and plant pathogens [107–109] developed via molecular marker assisted breeding or transgenic insertion of resistance-conferring genes are well known. However, molecular manipulation of crops for weed control has thus far been dominated by the development of herbicide-resistant crops [46]—a technology that requires all the economic, labor, and chemical inputs of conventional herbicide application.

Allelopathy has been reported in a number of crops but most current allelopathy research is concentrated on three grass species; wheat, rice, and sorghum [7,9]. Field trials comparing rice varieties with varying levels of allelopathy showed economic benefits from allelopathic varieties in terms of yield under weed competition and reduced need for herbicide, although the allelopathic varieties studied had commercially inadequate yield and grain quality [110]. Allelochemicals [111,112] and quantitative trait loci associated with allelopathy [113–115] have been identified in some crops, indicating that molecular marker assisted breeding might produce elite germplasm with high levels of allelochemicals. Genetic mapping has also identified homoeologous loci associated with allelochemicals across several grass species' genomes [116]. Functional genomics techniques [117–119] could also aid in the discovery of genes involved in allelopathy.

4.1.1. Allelopathy costs—grow or defend?

Can allelopathy be selected without yield loss? Ecological theory holds that plants can either grow or defend but not both [120]. However, there appears to be great variation in this phenomenon depending on the type of threat being defended against and the genetic background of the plant expressing the resistance trait [121]. Studies have shown defense traits that are independent of growth [122–124] and a survey of "grow or defend" literature [121] concluded that in many cases costs of resistance appeared to be due to linkage rather than pleiotropic effects. Thus, a genomics approach might reveal and break linkage disequilibrium with poor agronomic traits in crops where genetic diversity for allelopathy has been observed. In cases where pleiotropic yield costs are present, they would have to be weighed against the benefits derived from concomitant weed control. For example, allelopathic protection from parasitic weeds in a low-input system might prevent total crop loss, whereas in a high-input system benefits might be more modest (e.g. reduced herbicide use) and wouldn't necessarily offset a yield penalty.

4.1.2. Transgenic allelopathy

The allelochemical properties of sorghum are well known and whole-plant extracts have been used for economical weed control in subsistence wheat production [125]. An allelochemical exuded from sorghum root hairs, sorgoleone, has been extensively studied [9] and its biosynthetic pathway has been characterized [126]. Research is underway to identify genes encoding the enzymes involved in this pathway in anticipation of intraspecific transformation to bring increased allelopathy to elite sorghum germplasm or possibly interspecific transformation to a close relative such as rice that could produce sorgoleone with only minor modifications to an existing biosynthetic pathway [9]. Additional research seeks to better understand the physiology and genetics of root-hair allelochemical exudation in sorghum in hopes of applying such knowledge to the enhancement of allelopathy in crops [127].

Potential hurdles or drawbacks of transgenic allelopathy include autotoxicity when allelochemicals would be transferred to naïve species and the metabolic costs associated with allelochemical production sufficient to effectuate control [47]. The use of cultural practices such as intercropping or cover cropping could be limited and the prevention of gene flow to

related wild plants would have to be ensured (indeed, sorghum has two close relatives that are important weeds with which it readily interbreeds: shattercane and Johnsongrass). If there are weed species that are not affected by a given allelochemical, the system might simply serve to select for the proliferation of those weeds, as with herbicide tolerant weeds [128,129], although this risk could be weighed against potential benefits, such as effective control of a devastating weed such as witchweed.

In rotation systems with susceptible crops, volunteer allelopathic plants that arose in the rotation crop could become superweeds, inhibiting the crop plants growing nearby. Another concern in such cropping systems would be the persistence or half-life of the allelochemical in the soil. Could it last long enough to affect germination of the subsequent crop? At present, much remains unknown about the fate or persistence of allelochemicals in the soil or their effects on soil chemistry or microflora [7].

Other transgenic approaches to weed control are being pursued to create artificial allelopathy, particularly against parasitic weeds. Parasitic plants make physical contact with their hosts and may therefore be particularly susceptible to crop-produced protection schemes. Inducible transgenic expression of a gene encoding an antibiotic compound called sarcotoxin IA, taken from the genome of a flesh fly, has been tested in tobacco plants as a defense against parasitic weeds [130]. The toxin was expressed in the roots and induced by broomrape attack. Transgenic plants inhibited growth of broomrape plants but did not kill them or prevent them from reproducing. In addition, infested transgenic plants were stunted compared to plants that were not attacked. This model demonstrates that compounds whose original functions are not herbicidal may be useful in a weed control context, although improvements would be necessary to this particular system in order to achieve acceptable weed control.

4.2. RNA interference

RNA interference [131] technology has potential for protecting crop plants from parasitic weeds. RNA interference has been demonstrated to provide broad-spectrum, transgenic host–plant resistance to root-knot nematodes (*Meloidogyne* spp.) by interfering with a parasitism-specific gene [132] and a similar phenomenon was shown in protecting maize plants from feeding by corn rootworm beetle larvae [133]. RNA interference technology has been proposed for protection of maize from *Striga asiatica* [134]. To achieve this, double-stranded *S. asiatica* RNA mimics would be transgenically produced by the maize plant and would need to move from host to parasite via vascular pathways. Similar movement of host-produced macromolecules has been detected in another host–parasite plant system [135]. Such an approach would need to be developed on a case-by-case basis since a "broad-spectrum" approach against all parasitic plants using such technology is unlikely to succeed. A gene targeting plants in general could adversely affect the crop plant and genes that selectively target "parasitic plants" probably do not exist since parasitism in plants has arisen many times in evolution [136].

4.3. Suicidal cover crops

Cover crops, which have been used for generations to assist in weed control, have been proposed to gain an enhanced, more economical role with the aid of genetic manipulation [137]. Typically herbicides are used to kill cover crops in preparation for planting the principal crop, adding an expense to the practice of cover-cropping that can be significant depending on the system. However, with the insertion of a lethal gene construct that is inducible by an environmental or other external stimulus, the cover crop would wither in the field under conditions appropriate to the cropping system, such as the planting of the next crop in the rotation.

5. Manipulation of the target weed

5.1. kev genes

Direct manipulation of weed genomes to facilitate their own control has been proposed based on earlier proposals for insect control [138], including a series of highly creative and complicated systems tailored to debilitate certain weed targets based on their specific biological traits [10]. In general, these strategies rely on chemical induction of the debilitating genes to kill the weed and the genes used in these schemes were termed *kev* genes. The reliance on induction of *kev* gene expression to kill the weed makes this an inundative strategy, mainly applicable to high-input cropping or urban situations.

Spread of *kev* genes would occur via pollination of wild-type plants by transformed *kev*+ plants and would be accelerated through the use of a system of transposon-vectored transformation emulating the Transposons with Armed Cassettes-Targeted Insect Control Strategy (TAC-TICS) [138]. In the TAC-TICS system, genes are incorporated into active, multi-copy transposons, which are then transformed into the recipient genome. The multi-copy transposon construct enables the rapid spread of the introduced genes throughout naïve populations of the recipient species, as has been demonstrated in an insect model [139]. Plant transposons such as Ac/Ds [140] have been proposed to achieve this end in plant systems [10].

5.2. Transgenic female sterility

A system is proposed here that is similar to the above proposals [10] in that the target weed would be transformed with a construct designed to spread through a weed population by aid of a TAC-TICS-like [138,139] plant transposon vector. However, this proposal has more in common with the classical biocontrol model than the inundative model in that it is designed to spread a suicidal gene construct that would proliferate and disseminate through a target weed population and would not require any post-release assistance or induction for activation. It would effectuate rather than facilitate control of the target. The system is based on the creation and transformation into the target genome of a gene construct causing female-sterility in the weed. This female sterility would spread via pollen from female-sterile plants through successive generations by way of wild-type target plants, which would serve as the female parents. A similar concept, known as "daughterless," has been proposed for control of introduced carp in Australia [141], although the biology of that vertebrate system is obviously quite different from a plant system and it does not propose the use of a transposon-enhanced vector.

5.2.1. Female-sterile construct

The female-sterile construct would consist essentially of a promoter that is only active in a female reproductive organ (e.g. the stigma, style, or ovule) (see Fig. 1) driving a barnase [142] or similar gene that would kill any cell in which it was expressed. Other components that would be useful to the construct would be a reporter gene, such as one that could change leaf or flower color, making female-sterile plants readily identifiable in the laboratory as well as the field, and a chemically inducible [143] "kill switch" similar in concept to the *kev*-gene strategy mentioned above [10], which would allow for selective and rapid killing of female-sterile plants if desired. If for some reason the female-sterile construct

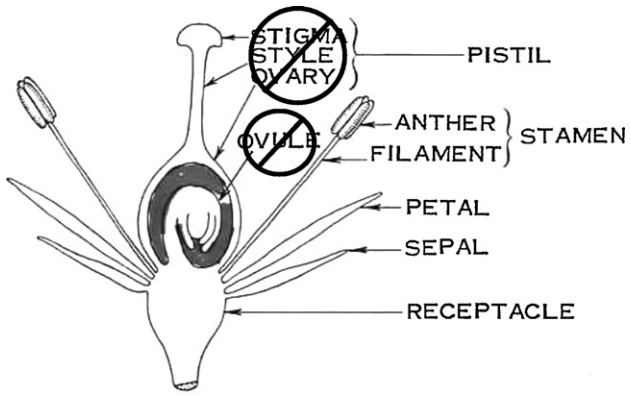

Fig. 1. Transgenic female sterility targets female flower parts. Female sterility would be accomplished by destroying one or more female reproductive organs using organ-specific promoters to express an RNAse or other destructive gene. Male reproductive organs would be unaffected, therefore the trait could spread via pollen to conspecific wild-type plants.

were disabled in the field, the deactivated female-sterile plants would simply revert to wild-type, perhaps with more active transposons than the average wild-type plant (which is unlikely to be a selective advantage [144,145]); they would cause no more harm than individuals from the extant invasive weed population that they were sent to infiltrate.

5.2.2. Specificity, spread, and seedbank replacement

Transgenic female sterility would be sexually transmitted by pollen and therefore would be highly specific to the target species, although attention would be necessary to the possibility of hybridization between the target and other closely related species. Target specificity may also be possible based on specificity of the promoter driving the female sterility gene, depending on how conserved the sequences of available promoters are [146]. The sexual nature of the spread of transgenic female sterility would turn the invasiveness of the weed against itself, spreading female-sterility as quickly as the weed spreads, through movement of both pollen and fertilized female-sterile seed. However, since female-sterile plants would require conspecific wild-type plants as "surrogate mothers" to reproduce, they could not spread by themselves to colonize areas where the target weed does not exist. Alone, they would produce only pollen, which would be effectively inert in the absence of wild-type target plants.

If the transgenic female-sterility strategy were to successfully eradicate an invasive weed population, the female-sterile seed left in the soil would gradually diminish, germinating to produce sterile plants until seed of neither female-sterile nor wild-type plants remained. Ultimately, the goal of transgenic female sterility is to gradually replace the seedbank of the target weed species with conspecific female-sterile seed. Ideally, in each successive post-release generation female-sterile plants would represent a steadily larger proportion of the overall target species population, competing with wild-type plants to pollinate wild-type flowers and filling the seedbank with a steadily higher proportion of female-sterile seed (see Fig. 2).

5.2.3. Multiple-locus female-sterile genotypes

Multiple releases might be necessary for a large weed population, as is often the case with classical biocontrol agents [1]. In the absence of an effective transposon-vectoring system, it would be possible to maximize the penetration of the female-sterile allele through a target weed population by including inducible deactivation of the female-sterile phenotype in the construct. This would allow several generations of pre-release interbreeding between female-sterile plants derived from independent transformation events to produce lines with multiple, unlinked copies of the female-sterile allele that would then form the release generation.

5.2.4. Limitations and target selection

Clearly, weed control by transgenic female sterility would only be feasible for out-crossing species that reproduce or spread primarily by seed. The target plant must also be amenable to transformation and preferably be susceptible to transposon activity. In addition, targets selected for such a project would likely be truly intractable weeds for which classical biocontrol and all other control measures had already failed (i.e. the "last line of defense"). Due to the reliance in transgenic female sterility on an out-crossing mode of reproduction in the target species, one possible result of such a release might be selection for self-pollinating plants in the target population. It could be argued that if

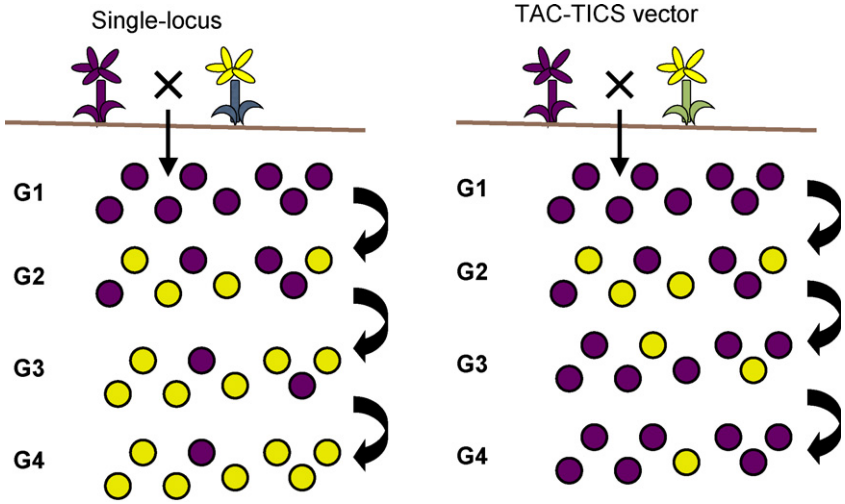

Fig. 2. Transposon vector increases penetration of the female-sterility gene construct through the target population. Assuming generation of homozygous female-sterile (FS) plants prior to release by crossing inducibly deactivated FS plants, first generation (G1) progeny of FS (purple) by wild-type (yellow) crosses would all be hemizygous for the FS allele. In subsequent progeny from a male parent containing a single locus of the FS allele (left), the frequency of the FS allele would decrease in the population by half with each generation. In progeny from a male parent with a TAC-TICS-like transposon vector (right; see Refs. [138,139]), FS allele frequency would increase in each subsequent generation leading to increasing proportions of FS seed in the seed bank.

the normal proportion of selfers is low in the wild population, the trait is not likely to be advantageous for the species in question, perhaps due to inbreeding depression. Indeed, selection for selfing would be particularly disadvantageous in autopolyploid targets [147].

Selection of a target, particularly for a first case, would be a nontrivial matter due to the potentially controversial nature of such a strategy. In addition to the biological parameters mentioned above, lack of hybridization with congeners would be an important consideration, as well as lack of proximity of the release site to the center of origin of the target species. The overall importance of the target weed to the society it affects might also figure into target selection. For example, a parasitic weed such as *S. hermonthica*, which has a devastating effect on subsistence farmers in Africa and which is known to primarily attack crop species, would seem to have no redeeming qualities and few, if any defenders [10,47]. Weeds that affect human health as well as agriculture, such as *Ambrosia artimesiifolia* and *Parthenium hysterophorus*, or illicit crops such as *Cannabis sativa* might also be reasonable choices. First, however, a test of the transgenic female sterility concept in a model plant species such as *Arabidopsis thaliana*, is warranted.

6. Concluding remarks

The discipline of biology has been revolutionized by discoveries and developments of molecular concepts and techniques. One of the greatest advantages of molecular biology is that it liberates us to think creatively and proactively in addressing challenges in biological systems, such as weed control. Molecular technology allows fine manipulation of the biological components of a weed management system, whether it is the agent, an affected crop, or the weed itself that is manipulated. Novel management strategies can take advantage of molecular advances in virtually any field of biology and apply them to weed control challenges.

Nevertheless, such advances do not obviate the concepts that predate them, such as classical biocontrol. In cases where classical biocontrol is promising for weed control, it should be encouraged, whereas in cases where existing management practices are insufficient and effective biocontrol agents are not forthcoming, other options, including molecular approaches, should be pursued. The need for invasive weed control research, both classical and molecular, is as urgent as ever and will only become more so.

Classical biocontrol and molecular approaches are not competing philosophies and there is no reason for mistrust between their practitioners. Classical biocontrol researchers are not dinosaurs and molecular geneticists are not Frankensteins [148]. They share the same goals, are regulated by the same authorities, and each promises to solve intractable weed challenges that the other cannot. Ultimately any solution, whether molecular or traditional, will need to be safe, efficacious, cost effective, and predictable if it is going to be adopted.

Some new technologies and proposals may work in certain situations and others will not. Some may need to be modified before they are practicable while others may be useful "right off the shelf." As the field of molecular biology matures, solutions to current challenges (e.g. prevention of gene flow out of transformed organisms) will be devised and perfected, as will new weed control strategies that might seem like science fiction today. (For example, parasitic weeds pose some of the most important weed challenges in the world today. Perhaps by studying these weeds or other parasitic plants, scientists could reverse the paradigm by finding the genes that enable plants to parasitize other plants. Moving such genes into crop species might enable the crops to parasitize the weeds, obviating the need for either herbicides or fertilizers.) Whatever the proposals, what is most important is that ideas be developed in the most open and transparent context possible. An informed and engaged public is more likely to welcome novel scientific solutions to challenges affecting them than a public that is kept at arm's length. The more information that is shared by scientists about their work, the less that work will be susceptible to sensational mischaracterization by the popular media. If such pitfalls can be avoided, both traditional biocontrol and molecular strategies will be considered by regulatory agencies based on their scientific merits alone, facilitating the decision-making process and advancing sustainable control of intractable, invasive weeds.

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